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PROSTAGLANDIN D_2 MODULATES HUMAN NEUTROPHIL INTRACELLULAR CALCIUM FLUX AND INHIBITS SUPEROXIDE RELEASE VIA ITS RING CARBONYL

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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We compared the effects of prostaglandin D_2 (PGD_2), prostaglandin F_2 alpha (PGF_2 alpha) and various ketones on O_2^- release by human neutrophils, which had been stimulated by N-formyl methionyl leucyl phenylalanine (FMLP). Our data suggests that the ring carbonyl of PGD_2 is essential to its inhibitory effect on O_2^- release, but the carbonyl group as a ketone, alone, is not sufficient. Using the fluorescent Ca^{2+} probe, Fura-2AM, we found that PGD_2 decreased the late phase of FMLP invoked changes in intracellular free Ca^{2+} ([Ca]i), but that PGF_2 alpha had no effect. Since cAMP altered FMLP stimulated [Ca]i in a pattern similar to PGD_2, we suggest that PGD_2 inhibits O_2^- release by increasing intracellular cAMP wiich in turn causes an increase in the rate of decline in [Ca]i. Furthermore, the ring carbonyl of PGD_2 is crucial to its effect on O_2^- as well as on [Ca]i.

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Prostaglandin D_2 (PGD_2) is the prostanoid which is most abundantly released by stimulated alevolar mast cells(1). Previously, we demonstrated that the release of O_2- by FMLP stimulated human neutrophils was inhibited by PGD_2. We found that PGD_2 was more potent than PGF_2 alpha, and suggested that the crucial determinant of potency was the ring carbonyl group on PGD_2. We also found that PGD_2 did not compete with FMLP at the FMLP receptor site (2).

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INTRODUCTION

In this present work, we tested this hypothesis that only the carbonyl group as a ketone was necessary for inhibition of O_2-. We also extended our understanding of the mechanism of O_2- inhibition by PGD_2 through observing the effect of PGD_2 on FMLP stimulated [Ca]i.

METHODS

Isolation of Neutrophils

Blood was obtained by venipuncture form healthy, medication-free volunteers. Preservative-free heparin (Gibco, Grand Island, NY) was used at a concentration of 12.5 units per ml of blood for anticoagulation. Erythrocytes were removed by gravity sedimentation through 2% dextran (Pharmacia, Upsale, Sweden) for 30 min. Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) was layered under the supernatant and centrifuged at 300 x g, room temperature for 10 min. Residual erythrocytes were removed by hypotonic lysis and the cells were centrifuged again at 150 x g. The neutrophil pellet was suspended in Hank's Balanced Salt Solution (HBSS, Gibco, Grand Island, NY) to the desired concentration. This procedure yielded 96% neutrophils by Wright's stain with a viability of at least 95% by trypan blue dye exclusion.

Assay of O_2-

The superoxide dismutase-inhibitable reduction of cytochrome C assay of Babior et. al. was employed (3). Neutrophils were preincubated for 15 min. with various concentrations of test reagents with HBSS as control. After preincubation, 500 μl of neutrophil suspension was added to the reaction mixture in 12 x 75 mm polystyrene tubes containing 1,500 μl HBSS, test reagents (250 μl) at various concentrations and 50 μm cytochrome C (Sigma, St. Louis, MO). The final cell concentration was 1 x 10^6/ml. After addition of

25 μl of FMLP at a final concentration of 10^{-6} or 10^{-7}M, or HBSS as control, the reactants were mixed and a 12 ml aliquot was placed into 12 x 75 mm plastic tubes which contained 10 μl superoxide dismutase (SOD: Boehringer Mannheim, W. Germany) at a final concentration of 50 μg/ml. All tubes were incubated at 37°C for 10 min. At the end of the incubation period 15 μl of DOS, also at a final concentration of 50μg/ml, was added to the other tubes to which SOD was not added initially. Centrifugation wa at 150 x g and the absorbance of the supernatant at 550 nm was determined. O_2- release was calculated as the difference in absorbance between the tubes which received SOD at the beginning, divided by an concentrations of PGD_2 (SIGMA, St. Louis, MO) was 95% or greater. Assays were performed in duplicate.

Measurement of Intracellular Calcium

5 x 10^6 cells were labelled with the membrane permeant probe Fura-2AM (Calbiochem) at 37°C for 30 min. at a probe concentration of 1 x 10^{-6} M. The cells were washed by centrifugation at 300 x g and resuspended in Hanks/HEPES. Fluorescent measurements were performed at 37°C in a Perkin Elmer Model 44B spectrophotofluoremeter. The cells were continually stirred during measurement. Alternative excitation wavelengths of 335 nm and 362 nm (slit 4 nm) were employed and emissions was measured at 510 nm (slit 8 nm). Calcium ion concentrations were calculated by the technique of Grynkiewicz et. al. (4) using the constants of Martell and Smith (5). FMLP control spectra were run prior to and after each experimental scan. Since our resting calcium levels determined with FURA-2AM were so much lower we investigated whether Quin-1AM gave us the same value for [Ca]i as did FURA-2AM. Where we had a resting

[Ca]i with FURA-2AM of 16 - 20 nM, with QUIN-2AM we had resting levels of [Ca]i from several individuals similar to those reported by others of 250-450 nM. Investigation with other cell lines gave similar results (manuscript in preparation, D.L. Mazorow and D.B. Millar).

Reagents

All reagents were prepared freshly each day. FMLP, prostaglandins, lipophilic ketones, and phorbol myristate acetate (PMA) were dissolved in dimethylsulfoxide (DMSO). Control solutions contained equal amounts of DMSO. Preliminary experiments showed no effect of DMSO on O_2- release (0.1 - 0.4%). The effect of PGD_2 upon the initial rate of reaction and the maximum O_2- released was measured as follows: The reaction mixture contained 3 x 10^6 cells/ml and 50 uM cytochrome C in a total volume of 3 ml. Preincubation was in plastic cuvettes at 37°C for 15 min. with various concentrations of test reagents. The cuvettes were then placed into a temperature-controlled dual beam spectrophotometer with a magnetic stirrer. FMLP at 10^{-6} or 10^{-7}M or PMA at 4 x 10^{-7}M or greater was added to one cuvette and control solution added to the other. The reaction was monitored until a maximum was reached, which for FMLP took 5-8 min. and with PMA took as long as 60 min., when low concentrations (4 x 10^{-9}M) were used.

Statistical Analysis

All experiments were of a paired design. The paired Student's t-test employed. $P < 0.05$ was considered significant.

RESULTS

Previous experiments showed no effect of PGD_2 on the release of O_2- generated by a cell-free xanthine-xanthine oxidase system (2).

In six experiments performed with neutrophils from six individuals a

biphasic dose response curve of the inhibition of O_2- release by PGD_2 was found (Fig. 1). The first phase occurred between 10^{-9} and 10^{-5}M and showed an inhibitory effect of up to 64%. In this concentration range PGD_2 had no effect on FMLP binding (2). In the second phase between 10^{-5} and 10^{-4}M, O_2- release was practically eliminated. This phase corresponds to the concentration at which PGD_2 decreases FMLP binding (2).

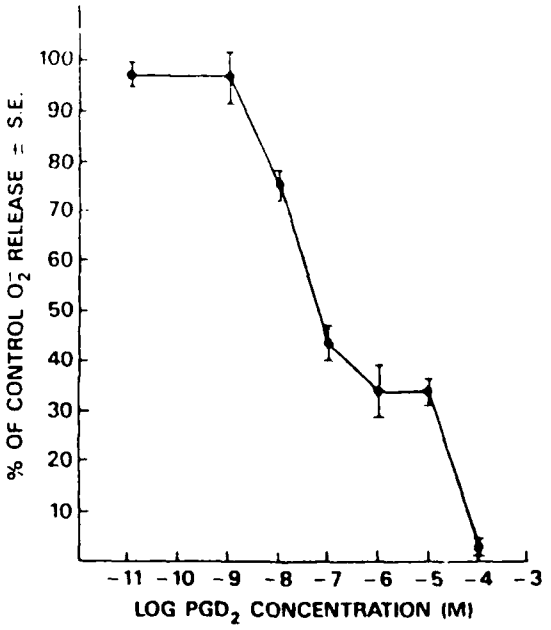

FIGURE 1.

Results are expressed as the percent of O_2- release +-S.E. in the absence of PGD_2. Unstimulated O_2- release is subtracted from all values. Each point is the average of six experiments performed with cells form six individuals.

$PGF_{2\alpha}$ is identical to PGD_2 except that $PGF_{2\alpha}$ has an hydroxyl group instead of a carbonyl group at the C11 position. The structures are shown in Fig. 2.

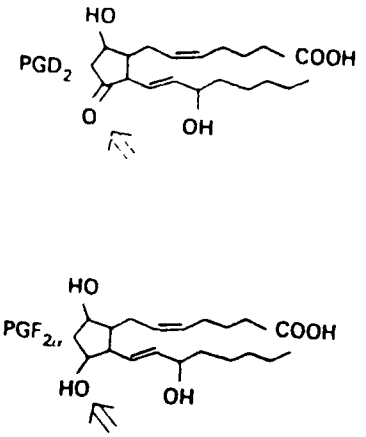

FIGURE 2.

The importance of the ring carbonyl was demonstrated by directly comparing the effect of PGD_2 to that of $PGF_{2\alpha}$ on O_2^- release (Table I).

TABLE I

Comparison of the Effect of 10^{-7}M PGD_2 and 10^{-7}M $PGF_{2\alpha}$ on O_2^- Release in Response to FMLP (10^{-7}M)

Subject	PGD_2	$PGF_{2\alpha}$	Ratio $PGD_2/PGF_{2\alpha}$
A	50.0	86.4	0.58
B	51.0	98.5	0.52
C	8.1	91.5	0.089
D	46.6	94.1	0.50
E	39.8	69.8	0.57
MEAN ± S.E.	39.1 ± 7.3	88.± 4.5	0.46 ± 0.9

$P < 0.005$ for significant difference of PGD_2 from $PGF_{2\alpha}$.

These results suggest that a major determinant of the inhibition of O_2- release by PGD_2 is the carbonyl group. Therefore, we studied the effects of various ketones and alcohols on FMLP stimulated O_2- release (Table II).

TABLE II

Effect of Ketones and Alcohols on O_2- Release

Reagent (10^{-6}M)	% of Control + S.E.
Cyclopentanone	98.4 ± 6.0
Cyclopentanol	88.5 ± 5.1
2,4 dimethylcyclopentanone	74.6 ± 7.8
Methanol	81.5 ± 4.0
Acetone	87.7 ± 6.8
PGD_2	54.5 ± 3.3
$PGF_{2\alpha}$	74.2 ± 5.3

10^{-6}M FMLP was used. O_2- release without alcohols or ketones was used as the control. Assays were performed on 2 to 4 individuals for each reagent. Included with each experiment was PGD_2 and $PGF_{2\alpha}$ at 10^{-6}M as controls.

None of the ketones or alcohols caused a marked decrease in O_2- release. Therefore, the carbonyl group alone as a ketone is not sufficient to inhibit O_2- release to a large degree. 2,4 dimethylcyclopentanone is more lipophilic than cyclopentanone and decreased O_2- release to 74% of control compared with 98% of control for cyclopentanone. This result suggests that increased lipophilicity is factor in the inhibitory effect of a ketone.

It is also of interest that in most cases PGD_2 inhibited O_2- release by approximately 50% regardless of whether PGD_2 and FMLP were used at 10^{-7}M to 10^{-6}M. For unknown reasons subject C of Table I was much more sensitive to PGD_2 than other individuals tested.

We found that PGD_2 decreased both the initial rate and the maximum quantity of O_2- release (Table III). PGD_2 alone caused no change in O_2- over the 15 min. preincubation period (data not shown).

TABLE III

Effect of PGD_2 (10^{-6}M) on the Initial Rate and Emax of O_2- Release Stimulated by 10^{-6M} FMLP.

Subject	Initial Rate	Emax
A	85.1	46.1
B	74.5	79.5
C	73.6	56.2
D	77.9	48.6
E	66.7	50.3
F	55.0	41.5
Mean ± S.E.	72.1 ± 4.21	53.7 ± 5.5

Control initial rate was from 2.82 to 9.33 mMoles/Min/1.5 x 10^7. Data are presented as percent of control cells. Control Emax was from 5.43 to 27.99 nMoles/1.5 x 10^7 cells.

The reduction of the initial rate of O_2- release by PGD_2 suggests that PGD_2 acted upon the rate limiting enzyme NADPH oxidase. This effect may be direct or indirect. In order to distinguish between these two possibilities, we determined the effect of PGD_2 on O_2- release stimulated with PMA. PMA activates NADPH oxidase via a pathway which is different from that FMLP. PMA

directly activates protein kinase C, thereby bypassing the FMLP receptor and other early signal transduction mechanism (6). We reasoned that if PGD_2 were directly acting upon NADPH oxidase then its inhibitory effect on O_2- release should be independent of the pathway of NADPH oxidase activation. We found that PGD_2 had no effect on PMA stimulated O_2- release even at PMA concentrations as low as 4×10^{-9}M. Lower PMA concentrations did not stimulate O_2- release. Therefore, PGD_2 is not acting directly upon NADPH oxidase (data not shown).

Next we examined the effect of PGD_2 on [Ca]i using the fluorescent dye Fura-2AM. PGD_2 had no effect on the initial rise in [Ca]i, after stimulation by 10^{-6} FMLP. However, in the PDG_2 treated cells, the rate at which [Ca]i decreased toward baseline was more rapid than in control cells (Fig. 3A). Since prostaglandins have been shown to increase cAMP levels (7), we determined whether cAMP may mediate the changes in [Ca]i caused by PDG_2. Incubation of cells with dibutyryl cAMP produced a change in rate of [Ca]i return to baseline similar to that of PGD_2 (Fig. 3). This experiment suggested that PGD_2 altered [Ca]i by altering cAMP levels. cAMP caused no change in the rate of return to baseline (data not shown).

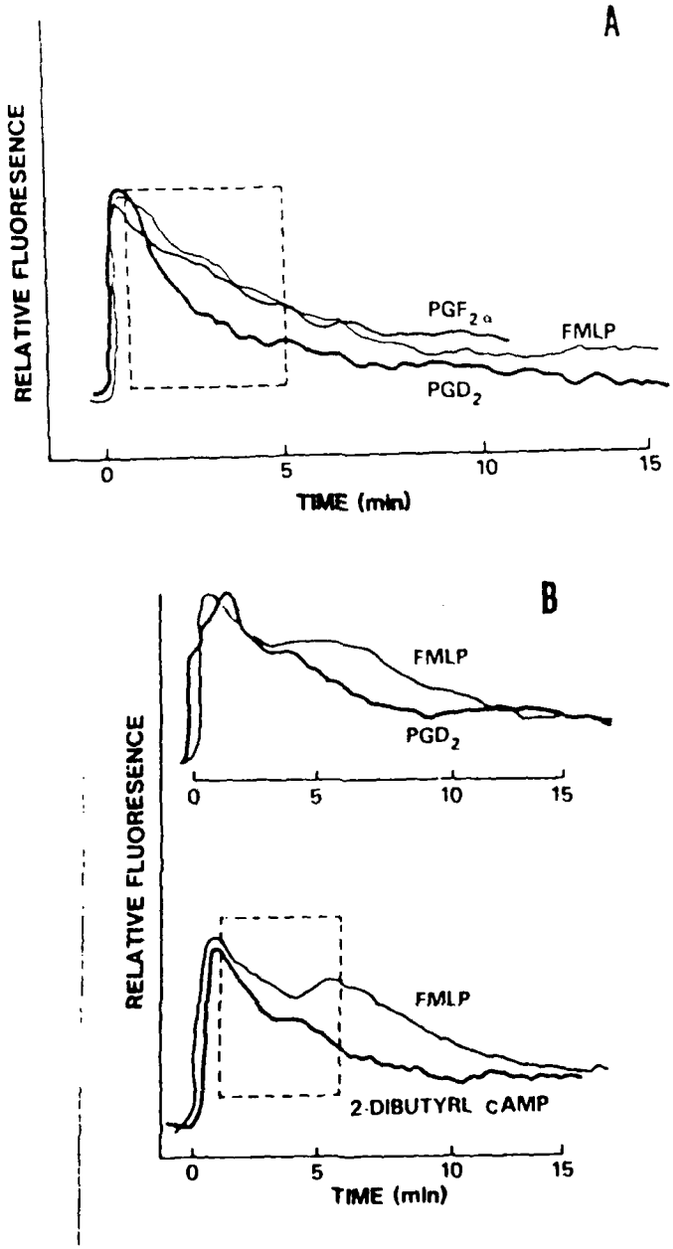

FIGURE 3.

A. Comparison of the effect of PGD_2 and $PGF_{2\alpha}$ on FMLP (10_{-6}M) induced changes in [Ca]i. Resting [Ca]i levels are 16-20 nM. and FMLP induced peak [Ca]i levels are 80-110nM.

B. The effect of 10^{-3} cAMP on changes in [Ca]i induced by 10^{-6} FMLP. All experiments were repeated three times with cells from at least three individuals and similar results were obtained.

DISCUSSION

Fanton and Kinnes (8) demonstrated that prostaglandins E_1 and I_2 inhibited FMLP stimulated O_2- release in human neutrophils. They also showed that $PGF_{2\alpha}$ had no effect on O_2- release (8). By comparing two prostaglandins which differed in only structural aspect, we showed that the carbonyl group of PGD_2 was essential to its inhibitory effect. The fact that a carbonyl as a ketone alone was not sufficient to inhibit O_2- release was shown by the ineffectiveness of the ketones. Lipophilicity of the ketone may be important in determining potency, as demonstrated by the increased effect of 2,4 dimethylcyclopentanone over that of cyclopentanone.

Our previous experiments showed that concentrations equal to and less than 10^{-5}M PGD_2 had no effect on FMLP binding (2). Therefore, PGD_2 is not acting via the FMLP receptor. Togni et al. showed that 10^{-5} cAMP caused a more rapid recovery of [Ca]i levels back to baseline but did not relate this observation to inhibition of O_2- release. Our results suggest that PGD_2 may inhibit O_2- release via cAMP. We found that cAMP but not cGMP causes the same changes in [Ca]i as does PGD_2. Furthermore, $PGF_{2\alpha}$ which had little effect on O_2- release also had no effect on [Ca]i. The experiments with PMA, in which PGD_2 had no effect even at a PMA concentration as low as 4×10^{-9}M showed that the PGD_2 interacts with the neutrophil in a carbonyl dependent manner to effect an increase in intracellular cAMP which enhances the recovery of kinase C and other kinases are Ca^{+2} dependent, it is possible that the decline in [Ca]i caused by PGD_2 leads to the inhibition of O_2- release (9). The lipophilicity rendered by the side chains of PGD_2 may allow the molecule to enter a critical

area of the cell membrane. The hydroxyl groups at C9 and C15 may be important in anchoring the prostaglandins into an important position by hydrogen bonding. Further work on studying the structure/function relationships among prostaglandins is clearly needed.

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